TraG Encoded by the pIP501 Type IV Secretion System Is a Two-Domain Peptidoglycan-Degrading Enzyme Essential for Conjugative Transfer

Karsten Arends,⁎a,Ertugrul-Kaan Celik,a,b Ines Probst,c Nikolaus Goessweiner-Mohr,⁎b Christian Fercher,b Lukas Grumet,b Cem Soellue,a,b Mohammad Yaser Abajy,a,b Tuuerkan Sakinc,d Melanie Broszat,⁎c,d Katarzyna Schiwon,a Guenther Koraimann,b Walter Keller,b Elisabeth Grohmann⁎a,c,d

Department of Environmental Microbiology/Genetics, University of Technology Berlin, Berlin, Germany⁎; Institute of Molecular Biosciences, University of Graz, Graz, Austria; Faculty of Biology, Microbiology, Albert-Ludwigs-University Freiburg, Freiburg, Germany⁎; Division of Infectious Diseases, University Medical Centre Freiburg, Freiburg, Germany⁎

pIP501 is a conjugative broad-host-range plasmid frequently present in nosocomial Enterococcus faecalis and Enterococcus faecium isolates. We focus here on the functional analysis of the type IV secretion gene traG, which was found to be essential for pIP501 conjugative transfer between Gram-positive bacteria. The TraG protein, which localizes to the cell envelope of E. faecalis harboring pIP501, was expressed and purified without its N-terminal transmembrane helix (TraGΔTMH) and shown to possess peptidoglycan-degrading activity. TraGΔTMH was inhibited by specific lytic transglycosylase inhibitors hexa-N-acetylmuramhexaose and bulgecin A. Analysis of the TraG sequence suggested the presence of two domains which both could contribute to the observed cell wall-degrading activity: an N-terminal soluble lytic transglycosylase domain (SLT) and a C-terminal cysteine-, histidine-dependent amidohydrolases/peptidases (CHAP) domain. The protein domains were expressed separately, and both degraded peptidoglycan. A change of the conserved glutamate residue in the putative catalytic center of the SLT domain (E87) to glycine resulted in almost complete inactivity, which is consistent with this part of TraG being a predicted lytic transglycosylase. Based on our findings, we propose that TraG locally opens the peptidoglycan to facilitate insertion of the Gram-positive bacterial type IV secretion machinery into the cell envelope.

Conjugation machineries of both Gram-negative (G−) and Gram-positive (G+) bacteria are classified as type IV secretion systems (T4SS), which function as translocators and mediate the transport of DNA and/or proteins across the cell envelope (1–3). Nevertheless, it is expected, due to the different architectures of the G− and G+ bacterial cell envelopes, that the mechanism of conjugation in G− and G+ bacterial systems may differ (2, 4–15).

The antibiotic resistance plasmid pIP501 from Streptococcus agalactiae is a model plasmid for conjugation in G+ bacteria and has a very broad host range for conjugative plasmid transfer and mobilization. Its host range includes virtually all tested G+ bacteria, including the multicellular filamentous streptomycetes and Escherichia coli (13, 16). The pIP501 transfer (tra) region encompasses 15 open reading frames (ORFs) that are organized in an operon negatively autoregulated by the first gene product TraA, a biochemically characterized conjugative relaxase (17, 18). The pIP501 tra gene products formerly named Orf1 to Orf15 have been renamed as TraA to TraO, as for the majority of the Tra proteins putative functions have been ascribed. TraE-psps01 is a putative VirB4-like ATPase, TraG-psps01 shows similarities to VirB1-like lytic transglycosylases (LTs), and TraL-psps01 is a putative VirD4-like coupling protein (2, 4, 19). Based on protein-protein interaction studies of the 15 pIP501 Tra proteins by yeast two-hybrid assays and in vitro pulldown assays, a first model of a T4SS-like system of G+ bacterial origin was proposed (4). Furthermore, in vitro ATP binding and hydrolysis were shown for both TraE-psps01 and TraL-psps01 (M. Y. Abajy and E. Grohmann, unpublished data) as well as binding of single-stranded DNA for the putative coupling protein TraJ-psps01 (K. Arends and E. Grohmann, unpublished data).

For the VirB1-like enzyme TraG, a modular architecture is anticipated (Fig. 1): at the N terminus of the protein, a putative transmembrane helix (TMH) is predicted which is followed by a soluble lytic transglycosylase (SLT) domain and an N-acetyl-d-glucosamine binding site (20, 21). LTs function as murein lyases by cleaving the β1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine (22–24). In addition to this, they also form a new glycosidic bond with the Cα hydroxyl group of the same muramic acid residue. Members are found in phages, type II and type III secretion systems, and T4SS (25). At its C terminus, TraG contains a C-terminal cysteine-, histidine-dependent amidohydrolases/peptidases (CHAP) domain corresponding to an amidase function. Many proteins with CHAP domains are involved in cell wall metabolism of bacteria; often, the CHAP domain is found in association with other pepti-
FIG 1 Domain composition of TraG. TraG shows a modular structure. The VirB1 ortholog TraG_{virB1} protein (GenBank sequence accession no. CAD44387.1) contains 369 amino acids. A transmembrane helix (TMH) is predicted at the N terminus (positions 20 to 36, HMMTOP), as well as a signal peptide with a putative cleavage site at positions 47 to 48 (SignalP 3.0). A specific lytic transglycosylase (SLT) domain is predicted at positions 80 to 165 (gray box); a cysteine-, histidine-dependent amidohydrolases/peptidases (CHAP) domain is putatively located at the C terminus (positions 243 to 369, white box). A peptidoglycan binding motif is predicted for the SLT domain (conserved domain search).

doglycan (PG)-cleaving domains. In these cases, the CHAP domain seems to have an endopeptidase specificity, thus opening the interlinks between several glycan strands (26, 27). The TraG CHAP domain also overlaps with an N-acetylmuramoyl-\(L\)-alanine amidase (PRK08581) domain (20, 21), indicating the putative cleavage of cross-bridges that interlink murein strands in PG (28).

The PG layer of the cell wall imposes structural constraints for the assembly of macromolecular secretion systems, such as the T4SS multiprotein complex. The structure of the PG must be rearranged to accommodate such structures, without compromising the integrity of the bacterial cell (29, 30). To overcome this barrier and to facilitate T4SS assembly, one or more PG lyases are generally encoded in the transfer regions of conjugative plasmids (29, 30). The PG lyases involved in locally opening the PG for the assembly of macromolecular secretion systems such as T4SS or type III secretion systems are designated specialized LTs (25, 31).

For G- bacterial systems, it was shown that VirB1-like proteins encoded by T4S genes are important, but some were found not to be essential for the T4SS to be functional, indicating that their function might be partially complemented by chromosomally encoded LTs (32–35). Transfer frequencies of the R1 plasmid were reduced between 5- and 10-fold in a gene homolog) deletion variant (31, 32, 35). virB1 mutants caused attenuated tumor formation in Agrobacterium tumefaciens because of only a limited number of known muramidases encoded on the G- bacterial chromosome. In these cases, the CHAP domain seems to have an endopeptidase specificity, thus opening the interlinks between several glycan strands (26, 27). The TraG CHAP domain also overlaps with an N-acetylmuramoyl-\(L\)-alanine amidase (PRK08581) domain (20, 21), indicating the putative cleavage of cross-bridges that interlink murein strands in PG (28).

A traG-knockout mutant showed that the protein is indispensable for plPF01 transfer between enterococci. In vivo localization of the TraG protein revealed an association of the protein with the Enterococcus cell envelope. TraG was expressed and purified without its TMH, and TraGΔTMH was demonstrated to possess PG cleavage activity on PG isolated from Enterococcus spp. and E. coli. Furthermore, we could demonstrate inhibition of TraG’s activity by bulgecin A and hexa-N-acetylchitohexaose, known inhibitors of LTs, suggesting that the proposed SLT domain was inhibited. There was a residual activity observed with the LT inhibitors which is expected and consistent with the presence of a functionally independent CHAP domain in TraG. In accordance with the proposed two-domain structure of TraG, both domains, when assayed independently, demonstrated PG-degrading activity.

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains used in this work are listed in Table 1. If not stated otherwise, all E. faecalis strains were grown in brain heart infusion (BHI) medium (Condalab, Madrid, Spain) at 37°C. BHI medium was supplemented, when required, with the following antibiotics: 50 \(\mu\)g fusidic acid (Fus)/ml and 20 \(\mu\)g chloramphenicol (Cml)/ml for E. faecalis JH2-2 (plPF01); 1 mg spectinomycin (Spect)/ml, 10 \(\mu\)g tetracycline (Tet)/ml, and 20 \(\mu\)g erythromycin (Em)/ml for E. faecalis C111 (pCF10-101, pKAΔtraG); 50 \(\mu\)g Fus/ml, 20 \(\mu\)g Cml/ml, and 100 \(\mu\)g gentamicin (Gent)/ml for E. faecalis OG1RF (plPF01, pKAΔtraG); and 1.5 mg streptomycin (Sm)/ml for E. faecalis OG1X. In the case of E. faecalis OG1RF (plPF01, pKAΔtraG), BHI medium was amended with 200 \(\mu\)g X-Gal (5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside)/ml. E. coli XL10 (Stratagene) and BL21-CodonPlus(DE3)-RIL (Stratagene) harboring pEU327 or derivatives thereof was grown in LB medium amended with 100 \(\mu\)g ampicillin (Ap)/ml and 50 \(\mu\)g Cml/ml at 37°C. E. coli DH5α (Invitrogen) harboring pEU327 or derivatives thereof was grown in LB medium amended with 100 \(\mu\)g Spect/ml, E. coli EC1000 (pCK147) was grown in BHI medium with 500 \(\mu\)g Em/ml, and E. coli EC1000 (pKAΔtraG) was grown in BHI medium supplemented with 20 \(\mu\)g Gent/ml.

Construction of a traG in-frame knockout mutant. A plPF01 traG in-frame deletion mutant was obtained using an allelic exchange method described by Kristich et al. (39) with minor modifications. First, the pSK41 Gent resistance gene uaaA-aphD encompassing both the promoter and terminator sequence (40) was amplified by PCR using primer pairs listed in Table S1 in the supplemental material. The PCR product was cut with BglII and cloned into plasmid pCK147/BglII, thus delivering the Gent resistance-encoding vector pKA.

Second, fragments of the plPF01 traG up- and downstream (1.017-bp and 1.038-bp, respectively) regions were amplified by PCR using primer pairs listed in Table S1 in the supplemental material and subcloned into plasmid pBlueScript SK− (Stratagene) via EcoRI/BamH1 and BamH1/I XbaI, respectively. The fused up- and downstream regions were cut with

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TABLE 1  Bacterial strains and plasmids used

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<sup>a</sup>Tetr, tetracycline resistance; Cmr, chloramphenicol resistance; Km, kanamycin resistance; Rif, rifampin resistance; Fusr, fusidic acid resistance; Spec, spectinomycin resistance; Smr, streptomycin resistance; Apr, ampicillin resistance; Gentr, gentamicin resistance; MLSr, macrolide-lincosamide-streptogramin B resistance; trα<sup>+</sup>, transfer proficient.

EcoRI/XbaI and inserted into EcoRI/XbaI-cut plasmid pKA, thus delivering pKAΔtrag. Subsequently, the conjugative strain E. faecalis CK111(pCF10-101) (39) was transformed with the suicidal vector pKAΔtrag by electroporation (41). pKAΔtrag was maintained in E. faecalis CK111 by P<sub>2</sub>rep4A4 encoded in trans on the chromosome.

To transfer pKAΔtrag from E. faecalis CK111(pCF10-101, pKAΔtrag) to the recipient E. faecalis OG1RF(pIP501), a biparental mating was performed. Transconjugants were selected on BHI medium supplemented with 50 μg Fus/ml, 20 μg Cm/ml, 100 μg Gent/ml, and 200 μg X-Gal/ml and screened for the integration of plasmid pIP501Δtrag into pIP501 at homologous sites with primer pairs listed in Table S1 in the supplemental material. Transconjugants in which pKAΔtrag had integrated up- or downstream of trag were grown on MM9YE agar supplemented with 10 mM l-3-glutaminolalanine (39) (Sigma-Aldrich) and subsequently screened for trag in-frame deletion by PCR. The trag in-frame deletion (pIP501Δtrag) was verified by PCR with flanking primer pairs binding outside the cloned region (see Table S1) and sequencing of the PCR products.

Western blot analysis of pIP501 T4SS proteins. E. faecalis OG1RF, OG1RF(pIP501), and OG1RF(pIP501 Δtrag) were grown at 37°C in BHI medium overnight. The cultures were centrifuged (4,000 × g, 10 min, 4°C), and the pellet was washed in 5 ml of potassium phosphate buffer (50 mM, pH 7.0) and resuspended in 0.75 ml lysis buffer (50 mM KH<sub>2</sub>PO<sub>4</sub> /K<sub>HPO</sub><sub>4</sub>, [pH 7.0], 1 mM EDTA, 1 mM MgCl<sub>2</sub>, 10 μl of 1-mg/ml DNase I). The cells were broken by sonication (30 mA intensity, 30 s with 0.5-s pulses; Sonopuls HD2070; Bandelin). The lysate was kept on ice for 30 min and centrifuged (4,000 × g, 5 min, 4°C) to remove unlysed cells. Fifteen microfilters of the supernatant was mixed with 6 μl of 4X SDS loading buffer. The samples were loaded onto 18% SDS-polyacrylamide gels, separated by electrophoresis, and blotted onto nitrocellulose membranes (Bio-Rad) using liquid transfer for 90 min at 90 mA (Mini Protean III system; Bio-Rad). The membranes were blocked in RotiBlock blocking solution (Carl Roth GmbH, Karlsruhe, Germany). The pIP501 T4SS proteins, TraH, TraK, and TraM, were detected through immunodetection with primary antibodies and a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Promega).

Complementation of pIP501 Δtrag. To complement the markerless trag deletion in trans, the expression vector pEU327 (42) was selected. trag was amplified from pIP501 with TraG_SalI-fw and TraG_SalI-rev primers. To amplify trag with its own ribosomal binding site (RBS), TraG_RBS_Sall-fw and TraG_Sall-rev primers were used (see Table S1 in the supplemental material). The 1,109- and 1,132-bp PCR products were cut with Sall and inserted into pEU327/Sall. The ligation mixtures were transformed into E. coli DH5α, Top10 (Life Technologies), XL10 Gold (Promega), and BL21 Star-pLysS (Life Technologies), respectively, and incubated at 37°C. With all the E. coli strains tested, only a few tiny colonies were obtained on selective agar plates (100 μg Spec/ml for pEU327 and 34 μg Cm/ml for E. coli BL21 Star-pLysS). No growth of putative transformants was obtained after inoculation into fresh selective solid or liquid medium, respectively, not even after incubation for up to 7 days. The cloning experiment with the trag wild-type gene cloned into pEU327 was repeated applying E. coli DH5α and One Shot Mach1-T1<sup>8</sup> chemically competent E. coli cells (Invitrogen) as host and testing distinct incubation temperatures for the selective plates, namely, room temperature, 30°C, and 37°C. Transformants were tested for the insertion of trag. None of them contained trag. Cloning of trag into the expression vector pMSP3353VA (43) also failed. Finally, trag without the putative TMH...
was amplified from pIP501 with TraG_{Δtmh}, Sall-fw and TraG_{Sall-rev} primers. The 1,013-bp PCR product was cut with Sall and inserted into pEU327/Sall. The ligation mixture was transformed into *E. coli* DH5α cells. Transformants obtained after 2 days of growth at 30°C were tested for the insertion of the *traG*ΔTMH fragment by sequencing with pEU327_Test_Sall-fw and pEU327_Test_Sall-rev primers (see Table S1). Plasmid DNA of pEU327-traGΔTMH was electroporated into *E. faecalis* OG1RF(pIP501)ΔtraG.

**Filter matings.** Overnight cultures were diluted 1:10 in fresh BHI medium and incubated until an optical density at 600 nm (OD_{600}) of 0.5 was reached. A 1:10 mixture of donor *E. faecalis* OG1RF(pIP501) and *E. faecalis* OG1RF(pIP501ΔtraG), respectively, and recipient (*E. faecalis* OG1X) cells were cultured on sterile nitrocellulose membrane filter (0.45 μm; Millipore). The membrane was incubated overnight cell-side-up on BHI agar at 37°C. Serial dilutions of the cells recovered by suspension in 1 ml phosphate-buffered saline (PBS) were plated on BHI agar supplemented with 1 mg Sm/ml to enumerate recipients and 1 mg Sm/ml and 20 μg Km/ml to enumerate transconjugants, respectively. To analyze complementation of the pIP501ΔtraG in-frame deletion in *trans*, matings were performed with *E. faecalis* OG1RF(pIP501ΔtraG), *E. faecalis* OG1RF(pIP501ΔtraG, pEU327-traGΔTMH), *E. faecalis* OG1RF(pIP501ΔtraG, pEU327), and *E. faecalis* OG1RF(pIP501) as donor, respectively, and *E. faecalis* OG1X as recipient. Transconjugants were selected on BHI agar supplemented with 1.5 mg Sm/ml, 20 μg Km/ml, and 20 μg Cl/ml. Recipients were selected on BHI agar amended with 1.5 mg Sm/ml and 2 mg Km/ml.

**Expression and purification of *TraG*ΔTMH, SLT_{TraG}, SLT(E87G)_{TraG}, and CHAP_{TraG} for biochemical studies.** All proteins were expressed as maltose binding protein (MBP) fusion proteins and purified as described in the supplemental material (see Fig. S1). Prior to application in the muramidase assay, they were concentrated to 0.7 mg/ml (TraGΔTMH) and to 5 mg/ml for the protein domains.

1-[3H]Lysine labeling of PG from *E. faecalis*. Five milliliters of an *E. faecalis* JH2-2 overnight (on) culture was transferred to 100 ml Todd-Hewitt broth (THB) (supplemented with 25 μg rifampin [Rif]/ml). One milliliter 1-[3H]lysine (1 mCi) was added, and the culture was grown at 37°C and 225 rpm to an OD_{600} of 0.5. Cells were harvested (7,000 × g, 15 min, 4°C) and washed with 8 ml 25 mM Tris-HCl (pH 8.0) at 4°C. The pellet was vacuum dried and suspended in 8 ml 4% SDS. The following steps were performed as described under “PG isolation and purification” in the supplemental material. The labeled PG was lyophilized and stored at –80°C.

2.6-[3H]Diaminopimelic acid labeling of PG from *E. coli*. Five milliliters of an *E. coli* DH5α of an overnight culture was transferred to 100 ml M9 minimal broth, and 1 ml 2.6-[3H]diaminopimelic acid (1 mCi) was added. Incubation at 37°C continued until stationary phase was reached (OD_{600} of ca. 1.0). The protocol continued as described under “PG isolation and purification from *E. coli* DH5α” in the supplemental material.

**Muramidase assay.** The muramidase assay is based on the measurement of the solubilization of 1-[3H]lysine-labeled PG from *E. faecalis* and 2.6-[3H]diaminopimelic acid-labeled PG from *E. coli* (31). Labeled PG (approximately 5,000 cpm) was incubated with 3 μM respective protein in a total volume of 100 μl 20 mM Bis-Tris buffer (pH 5.3) for 30 min at 37°C. One percent ceteryltrimethylammonium bromide (CTAB) was added to precipitate the insoluble substrate. Samples were kept on ice for 30 min. After centrifugation (4 min at 17,000 × g and 4°C), 160 μl of the supernatant was added to 7 ml scintillation cocktail (Jonophor Gold scintillation cocktail; Fuji, Tokyo, Japan). Radioactivity was measured using the Wallac 1409 liquid scintillation counter (PerkinElmer). Purified MBP and lysozyme from hen egg white were used as negative and positive controls, respectively.

**Cy3 labeling of PG.** Cy3 labeling of PG was performed according to the method of Zähr et al. (31) with modifications. Five hundred microliters PG from *E. faecalis* JH2-2 (1 mg PG in 500 μl distilled water) was incubated with 50 μl 1 M borate buffer (pH 9.6) and 5 μl Cy3-N-hydroxy succinimide (NHS) ester (1 mg/ml in dimethyl formamide) for 60 min at room temperature. Excess Cy3 was removed by centrifugation (5,000 × g, 5 min at 4°C). Cy3-labeled PG was stored at −20°C.

**Cy3 spot assay.** The Cy3 spot assay was performed according to the method of Zähr et al. (31) with modifications. Eight-well glass slides were coated with poly-L-lysine (Sigma-Aldrich) for 30 min at room temperature, rinsed with deionized water, and dried. Ten microliters deionized water and 1 μl Cy3-labeled PG (containing approximately 2 μg PG) were spotted onto the poly-L-lysine-coated wells and incubated at room temperature for 45 min in the dark. The slides were rinsed with deionized water for 2 min, dried at room temperature in the dark, and scanned with a GenePix 4000B microarray scanner with 532-nm excitation (GenePix Pro 4.1 software). Ten microliters Bis-Tris buffer (pH 5.3) was loaded onto the PG, and approximately 6 pmol of the respective protein, lysozyme, *TraG*ΔTMH, and MBP, was added. The slides were incubated at 37°C for 2 h 30 min. To remove the digested material, the slides were rinsed with deionized water for 2 min and dried at room temperature. To quantitate the fluorescence signals, dry slides were scanned before and after incubation with the enzyme using an array scanner (Axon GenePix 4000B; photomultiplier tube [PMT] setting, 320; scan power, 33%,) and images were obtained and analyzed using the GenePix Pro 4.1 program. Line scans from the images were produced using a 50-pixel (2-mm) window across the center of the PG spots. Relative PG-degrading activity of *TraG*ΔTMH was calculated using the following formula: 100 – ([FI532b/FI532a] × 100), where FI532a is the mean fluorescence intensity of Cy3-labeled PG, corresponding to a total of 460 pixels in a circle with a diameter of 1 mm, before incubation and FI532b is the same value after incubation, respectively.

To test inhibitory effects of the specific lytic transglycosylase blockers bulgicin A (Hoffman-La Roche) and hexa-N-acetylchitosaxohe (Seikagaku, Japan) on the PG hydrolysis activity of *TraG*ΔTMH, the Cy3 spot assay was repeated with the exception that prior to addition of the respective protein the lytic transglycosylase blockers were added in concentrations ranging from 0.5 to 4 mM for hexa-N-acetylchitosaxohe and 1 to 20 mM for bulgicin A. Data analysis and calculation of relative PG-degrading activities were done as described above.

**Subcellular fractionation of *E. faecalis* JH2-2 (pIP501) and immunolocalization of *TraG*.** Subcellular fractionation of *E. faecalis* JH2-2 (pIP501) was performed according to the method of Buttarro et al. (44) with modifications. Briefly, *E. faecalis* JH2-2 (pIP501) was grown at 37°C in BHI medium to an OD_{600} of 0.5. The culture was chilled on ice for 15 min, washed twice in an equal volume of potassium phosphate buffer (50 mM, pH 7.0), and resuspended (1:50 [vol/vol]) in lysis buffer (50 mM KH_{2}PO_{4}/K_{2}HPO_{4}, pH 7.0, 1 mM EDTA, 1 mM MgCl_{2}, 100 μg/ml DNase, 100 μg/ml RNase). The cells were broken by FastPrep-24 (MP Biomedicals) using lysing matrix E (1.4-mm ceramic spheres, 0.1-mm silica spheres, and 4-mm glass beads; MP Biomedicals). The lysate was centrifuged (1,500 × g, 20 min, 4°C) to remove unlysed cells. The supernatant was transferred and centrifuged at 17,000 × g for 20 min at 4°C to collect the cell wall fraction. Membranes were harvested by ultracentrifugation of the supernatant at 163,000 × g for 2 h at 4°C (50 Ti rotor; OTT Combi ultracentrifuge; Thermo Fisher Scientific GmbH) and resuspended in 50 mM KH_{2}PO_{4}/K_{2}HPO_{4}, pH 7.0, 1% Triton X-100. The remaining supernatant contained the cytoplasmic fraction of proteins.

Subsequently, equal amounts of the cell wall, membrane, and cytoplasmic fraction were applied onto SDS-polyacrylamide gels, separated by electrophoresis, and blotted onto nitrocellulose membranes (Bio-Rad) using liquid transfer for 90 min at 90 mA (Mini Protean III system; Bio-Rad). The membrane containing the transferred proteins was initially blocked in RotiBlock blocking solution (Carl Roth GmbH, Karlsruhe, Germany). *TraG* was then localized in the fractions (cell wall, membrane, and cytoplasm) by immunostaining of *TraG* with primary polyclonal anti- *TraG*ΔTMH antibodies and a secondary HRP-conjugated anti-rabbit IgG antibody (Promega).
RESULTS

TraG is an essential T4SS protein. The indispensability of VirB1 and homologous proteins could not be demonstrated for T4SS originating from G− bacteria (31–35). However, due to the multilayered and cross-linked PG meshwork in G+ bacteria, we raised the question whether a specific PG degradation caused by the putative lytic transglycosylase TraG might be a crucial step during conjugative pIP501 DNA transfer in G+ bacteria. To investigate the potential indispensability of TraG in conjugative transfer, we constructed a pIP501ΔtraG in-frame deletion mutant using the PhoS counterselection markerless exchange system (39). Ninety-seven percent of the TraG coding region was thereby deleted, except for the first 5 N-terminal codons and the last 6 C-terminal codons, to not alter transcription and translation of downstream tra genes.

We assessed the influence of the traG deletion on conjugative transfer of pIP501 by biparental matings. Assuming the indispensability of TraG, no transfer should occur in the traG-knockout mutant in a biparental mating in G+ bacteria, whereas transfer rates with an isogenic, traG-proficient pIP501 plasmid should be in the expected range of approximately 10−3 transconjugants per recipient. The biparental matings were performed with donor strains E. faecalis OG1RF(pIP501ΔtraG) and isogenic E. faecalis OG1RF(pIP501), respectively, and E. faecalis OG1X as recipient.

The isogenic E. faecalis OG1RF(pIP501) originated from the same E. faecalis OG1RF strain that harbored the merodiplod pIP501-pKAΔtraG complex. Both the traG-knockout plasmid pIP501ΔtraG and pIP501 used for the biparental mating assay were obtained after excision and segregation of the suicide vector pKΔtraG from the pIP501-pKΔtraG complex.

Transfer rates of the traG deletion mutant were below the detection limit of the assay (<3.5 × 10−9 ± 2.4 × 10−9, mean value of three independent assays), whereas transfer rates with the isogenic strain E. faecalis OG1RF(pIP501) were at least 20,000 times higher and in the expected range for pIP501 of 7.6 × 10−5 ± 5.7 × 10−5 transconjugants per recipient cell (mean value of three independent assays). As transfer frequencies were in the expected range for pIP501, we can exclude polar effects due to an unwanted suicide vector integration into pIP501. Unfortunately, we were not able to complement the traG knockout in E. faecalis pIP501ΔtraG by supplying the traG wild-type (wt) gene on an expression plasmid in trans. Despite several attempts, it was not possible to clone traG in E. coli/G+ bacterial shuttle vectors. A possible explanation is that TraG is toxic in E. coli even if basal expression is very low. In the natural plasmid context, expression of all pIP501 tra genes is tightly controlled by the first gene product of the tra operon, TraA (18). Furthermore, we tried to complement the traG deletion by supplying traGΔTMH in trans. We obtained transformants of pEU327-traGΔTMH in E. coli, and the plasmid DNA was subsequently electroporated into E. faecalis OG1RF(pIP501ΔtraG). However, transfer activity of pIP501 could not be restored by supplying traGΔTMH in trans.

A complete transfer deficiency was observed for the traG-knockout strain (E. faecalis pIP501ΔtraG) and the complementation strain (E. faecalis pIP501ΔtraG, pEU-327-traGΔTMH), as well as for the negative control (E. faecalis pIP501ΔtraG, pEU-327), in three independently performed assays. Transfer frequencies of pIP501 were in the expected range of approximately 5 × 10−5 transconjugants/recipient under the same conditions.

To ensure that the excision of traG by allelic exchange did not alter transcription and translation of downstream tra genes in pIP501ΔtraG that could have caused the failure of complementation, we performed immunoblot assays with protein lysates obtained from E. faecalis OG1RF(pIP501) and E. faecalis OG1RF(pIP501ΔtraG). Three genes located downstream of traG in the pIP501 tra operon were selected for immunodetection with the respective polyclonal anti-Tra antibodies, namely, traH, traK, and traM. The expression level of all three genes was unaltered in the traG-knockout mutant (Fig. 2), proving that deletion of 97% of the traG coding region exerts no negative effect on expression of the downstream genes in the pIP501 tra operon.

Therefore, the inability of TraGΔTMH to complement the traG knockout could most probably be caused by an incorrect folding of TraGΔTMH due to the missing putative TMH or by a mislocated domain of TraGΔTMH within the enterococcal cell or both.

TraG localizes to the E. faecalis cell envelope. T4SS of G− bacteria are multiprotein complexes that span the cell envelope (6, 7). It is likely that, due to its indispensability for conjugative pIP501 transfer and its predicted PG-degrading activity, TraG localizes within the cell envelope. In silico predictions postulated TraG being predominantly localized in the cell wall fraction (PSORTb v.3.0.2 [45]) with a TMH (amino acid [aa] positions 17 to 36, CAD44387, HMMTOP [46]) and a possible signal peptide (putative cleavage site between aa 47 and 48, CAD44387, SignalP3.0 [47]). To localize TraG in vivo, an exponentially growing culture of E. faecalis JH2–2(pIP501) was fractionated into cell wall, membrane, and cytoplasmic fractions according to the method of Buttar et al. (44) with modifications. As expected, TraG was exclusively found in the cell envelope fractions (cell wall and membrane, Fig. 3A). Other results from our lab confirmed that the cell wall fractions were not contaminated with cytoplasmic proteins, since the pIP501 Tra protein TraN, under the same conditions, was exclusively found in the cytoplasmic fraction (Fig. 3B). This is in agreement with the in silico prediction for the cytoplasmic localization of TraN in G+ bacteria (N. Goessweiner-Mohr, K. Arrends, E. Grohmann, and W. Keller, unpublished data).

TraGΔTMH degrades peptidoglycan and is inhibited by hexa-N-acetylchitohexaose and bulgecin A. To demonstrate the sequence-inferred PG-degrading activity of TraG in vitro, we performed an assay as described by Zahrl el et al. (31) using Cy3-labeled PG from E. faecalis JH2–2. PG-hydrolyzing activity was calculated by comparing fluorescence levels of Cy3 PG spots on a glass surface before and after enzyme treatment (for details, see Materials and Methods). To visualize PG degradation, line
scans through the PG spots before and after treatment were superimposed (Fig. 4). PG degradation was observed in the case of TraG/H9004TMH and lysozyme (99%), whereas for purified MBP, which was used as a negative control in all of the performed assays, no or only minimal background activity (as in this case, 15%) was seen (Fig. 4A).

To investigate a possible lytic transglycosylase activity of TraG/H9004TMH, we repeated the Cy3 spot assay in the presence of the specific lytic transglycosylase blockers hexa-N-acetylchitohexaose and bulgecin A, respectively. For hexa-N-acetylchitohexaose, which binds to the active center of LTs but is not a substrate for these enzymes (48–50), concentrations from 0.5 mM to 4 mM were tested: the addition of 4 mM inhibitor reduced TraG/H9004TMH activity, resulting in a residual activity of 27% (Fig. 4B). For bulgecin A, concentrations between 1 mM and 20 mM were added (Fig. 4C). Concentrations of 5 to 20 mM bulgecin A resulted in 21 to 25% residual TraG/H9004TMH activity (no PG degradation occurred with the MBP control in this experiment; data not shown). Since bulgecin A, a specific glycopeptide inhibitor of LTs (51), is known to bind to the active site of LTs and is frequently used as a ligand in crystallographic studies revealing the structure and function of LTs (52, 53), our data suggest binding (at 5 mM bulgecin A) of the inhibitor to the predicted SLT domain, leaving the CHAP domain uninhibited. To accurately quantify PG degradation activity of TraGATM/H and its domains, a radioactive muramidase assay was performed.

TraG SLT and CHAP domains degrade PG independently of each other. To further analyze whether the residual TraGATM/H activity correlates with a putative PG-degrading activity of the CHAP domain, we expressed and purified both domains independently. PG hydrolase activity of TraG/H9004TMH and its N-terminal SLT and C-terminal CHAP domain was investigated by a radioactive muramidase assay in which solubilized cell wall components were measured with two different substrates (PG from G+/ and G− bacteria, respectively). TraGATM/H showed high muramidase activity (1,956.6 cpm after subtracting background activity of 215.5 cpm) when using L-[3H]lysine-labeled PG from E. faecalis JH2-2 and was comparable to the activity of lysozyme from hen egg white (2,438 cpm) under the same conditions. SLTTraG still exhibited 46% (900.07 cpm) of TraG/H9004TMH enzyme activity (1,956.6 cpm), and CHAPTraG exhibited 34% of TraG/H9004TMH activity. A single-amino-acid change in the putative catalytic center of TraGSLT (E87G) resulted in a 74% reduction of muramidase activity as measured for the SLTE87G domain in comparison to SLT (Fig. 5).
DISCUSSION

In this work, we demonstrated the essential function of the VirB1-like PG hydrolase TraG in conjugal plasmid transfer of broad-host-range plasmid pIP501. To the best of our knowledge, this is the first time that indispensability of a VirB1-like PG hydrolase could be shown for T4SS-mediated DNA/protein transport. Bantwal and coworkers constructed deletion mutants of the pCW3 virB1 homolog, tcpG. The pCW3ΔtcpG mutants showed ca.-1,000-fold-reduced conjugal plasmid transfer capacity (30) whereas the pIP501ΔtraG variant did not show any residual transfer activity in intraspecies transfer between different E. faecalis strains. Bantwal et al. (30) explained the residual pCW3 transfer activity by putative functional redundancy between PG hydrolases produced by C. perfringens, with chromosomally encoded enzymes able to catalyze the PG degradation required for conjugal transfer, albeit at lower efficiency. For E. faecalis, only scarce information on genomic PG hydrolases is available; most literature deals with the two autolysins, AtA and Atn, and their role in autolysis of E. faecalis cells as well as in DNA-dependent Enterococcus biofilm development (54–56). No putative role in controlled local opening of the PG has been proposed for these enzymes.

Both TraG domains, the SLT_TraG and the CHAP_TraG domains, have been expressed separately and shown to possess PG degradation activity on PG from E. faecalis (Fig. 5). PG degradation activity of the SLT_TraG domain is in agreement with lytic transglycosylase activity of several putative lytic transglycosylases encoded by G− bacterial T4SS, P19 from the conjugative resistance plasmid R1, VirB1 encoded by the Ti plasmid of A. tumefaciens, TcpG from the small chromosome of B. suis, and HP0523 encoded by the cag pathogenicity island of H. pylori (31); AtA from the Neisseria gonorrhoeae T4SS-secreting DNA (57); and TcpG encoded by the G+ bacterial T4SS from C. perfringens plasmid pCW3 (30).

We mutated the putative catalytic residue of SLT_TraG, the glutamate at position 87 (GenBank sequence accession no. CAD44387.1), by replacement with glycine, generating SLT(E87G)_TraG and introducing a BamHI restriction site to facilitate screening for mutants. Surprisingly, the mutant protein retained about 26% of its LT activity compared to SLT_TraG. Supposedly, the mutated catalytic active glutamate at position 87 (GenBank CAD44387.1) in TraG could be partially complemented by a second putative ES motif at positions 100 to 101 (GenBank CAD44387.1) (positions 48 to 49, HMM logo, http://pfam.sanger.ac.uk/family/PF13702.1#tabview=tab4 [58]), thus explaining the strongly reduced, but not completely abolished, activity of SLT(E87G)_TraG.

TraGATMHH PG-degrading activity was significantly reduced by the specific lytic transglycosylase inhibitors bulgencin A and hexa-N-acetylchitohexaoase (48, 49, 51), suggesting a possible lytic transglycosylase activity for TraG. For IgpF, the lytic transglycosylase from the plasmid-encoded type III secretion system of Shigella sonnei (59, 60), a similar observation was made with hexa-N-acetylchitohexaoase. It completely inhibited IgpF at a concentration of 4 mM. Zahrl et al. (31) showed that, in contrast, lysozyme was not affected by the presence of hexa-N-acetylchitohexaoase, which is in accordance with the observation that this substance is not a substrate for lysozyme and solely inhibits lytic transglycosylases (48, 49).

Zahrl et al. (31) also tested bulgencin A, which has also been described as an inhibitor of lytic transglycosylases (51), and found that a concentration of 20 mM was sufficient to completely inhibit IgpF. TraGATMHH, however, was not totally inhibited by bulgencin A. Its activity was reduced to around 21 to 25% residual activity at 5 mM and higher (up to 20 mM) bulgencin A concentrations, which could be due to binding of bulgencin A to the SLT domain but not to the CHAP domain. This observation is consistent with the SLT and CHAP domains of TraG representing independent domains, with both displaying PG-degrading activity.

TraGATMHH was also able to degrade 2,6-[^3H]diaminopimelic acid-labeled PG from E. coli DH5α, albeit with less activity. For TraGATMHH, 2,398 cpm of soluble PG fragments was measured; for lysozyme, we obtained 6,712 cpm, thus resulting in 38% activity for TraGATMHH compared to lysozyme. These data are in agreement with the broad host range of pIP501, which was, in addition to self-transfer to virtually all Gram-positive bacteria, shown to transfer in and be stably maintained in E. coli (16). The reduced TraGATMHH PG degradation activity observed for E. coli PG might be compensated for by other LTs encoded on the E. coli chromosome.

Our data strongly suggest that TraG is a PG-degrading protein that is indispensable for the intraspecies conjugative transfer of pIP501 in E. faecalis. To the best of our knowledge, it is the first time that indispensability of a VirB1-homologous protein has been shown for conjugal plasmid transfer.

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